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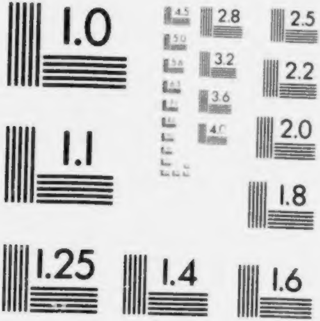

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# ON THE REMOVAL OF DIFFUSIBLE SUBSTANCES FROM THE CIRCULATING BLOOD OF LIVING ANIMALS BY DIALYSIS

## II. SOME CONSTITUENTS OF THE BLOOD

BY

JOHN J. ABEL, L. G. ROWNTREE AND B. B. TURNER

*From the Pharmacological Laboratory of the Johns Hopkins University*

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# ON THE REMOVAL OF DIFFUSIBLE SUBSTANCES FROM THE CIRCULATING BLOOD OF LIVING ANIMALS BY DIALYSIS

## II. SOME CONSTITUENTS OF THE BLOOD

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*From the Pharmacological Laboratory of the Johns Hopkins Medical School*

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I. In an earlier paper[1] the authors have described in detail their method of removing diffusible substances from the blood of a living animal. Essentially the method consists in the continuous passage of the blood from an artery through a system of tubes made of celloidin immersed in a saline solution or artificial serum, and its return into a vein, thus yielding by dialysis certain of its constituents to the fluid surrounding the tubes. Improvements in the apparatus are still being made and will be described later.

We desire at present, 1, to present certain data to show that the new method enables the investigator to accumulate the various non-protein constituents of the blood in any desired amount, the quantity possible to be obtained depending on the dialyzing surface of the apparatus and the number of experiments made; 2, to summarize briefly the results obtained by us in the isolation of definite substances.

In addition to the smaller quantities collected up to May, 1913, from which were obtained the preliminary results reported in our first paper (loc. cit., p. 315), diffusible constituents of the living blood have now been collected for a total of 112 hours with the apparatus described on pp. 287–291 of that paper. The total diffusing surface of the thirty-two tubes in this apparatus is about 3200 square cm., the volume of blood varied from about 800 to 500 cc. according to the extent to which the tubes were flattened by external pressure and the dialysate, which was changed hourly, measured 3 liters. The animals used were dogs varying in weight from 11 kgm. to 34 kgm., average 17 kgm. The average duration of an experiment was 7 hours,[2] while the longest lasted for almost 18 hours.

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[1] This Journal, vol. v, pp. 275–316, 1914.

The total quantity of nitrogen obtained in the 112 hour diffusate was in the neighborhood of 20 grams. This estimate is based on an analysis of the material obtained in the first 74 hours which showed 12.5 grams in the filtrate from the phosphotungstic acid precipitation besides about 1 gram in the precipitate after decomposition and small quantities lost in the course of the chemical procedure; and also on a 5-hour quantitative experiment in which the determination of the total nitrogen in the diffusate by Kjeldahl's method gave an average of 0.170 gram per hour.

A large part of the above consists of urea, as is shown in the following paragraph, while smaller amounts of ammonia and other nitrogenous substances are lost in evaporation, etc., and about 1½ to 2 grams are separated by phosphotungstic acid. In the final alcoholic solution of the amino-acids and other substances after esterification there was found, excluding some urea and ammonia which had not been completely removed, 3.73 grams of nitrogen of which 1.5 grams[3] was amino-nitrogen by Van Slyke's test. This would indicate the presence of about 12 to 16 grams of mono-amino-acids besides the bodies giving the $\alpha$-amino-acid reaction in the phosphotungstic precipitate. The amounts isolated have been much smaller, for reasons given elsewhere.

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[2] Two or three dogs succumbed early for various reasons of an accidental character: disregarding these the average would be at least an hour longer.

[3] In our previous paper (loc. cit., p. 315) we stated that "at least 5 grams of amino-nitrogen exclusive of urea" was in our hands. This was an over-estimate, based on the mistaken assumption that the nitrogen of the amino-acids corresponded closely in amount to the difference between the total non-proteid nitrogen and that of the urea and ammonia, neglecting the large amount of creatine, etc., present in the blood.

The amounts of diffusible substances obtained per hour with a given apparatus appear to vary but little with the rate of flow of the blood through it, and therefore, indirectly, with the size of the animal used. Thus in the above mentioned 5-hour experiment, the composition of the diffusate, which was changed hourly, was in the

| | |
|---|---|
| 1st hour | 52.4 mgm. N per liter |
| 2nd and 3rd hours (mean) | 57.7 mgm. N per liter |
| 4th and 5th hours (mean) | 59.1 mgm. N per liter |

The rapidity of the flow decreased considerably during the experiment, in spite of which the strength of the diffusate increased, although the difference is too small to have much significance.

The determination of urea by Marshall's method and of preformed ammonia showed a similar constancy, the former varying from 43.5 to 45.4 mgm. per liter (i.e., from 20.3 to 21.2 mgm. urea-nitrogen), the latter from 1.8 to 2.6 mgm. (1.5 to 2.2 mgm. nitrogen).

It is noticeable that the proportions of the constituents determined in the diffusate follow approximately those of the same substances in the blood[4] (see, e.g., Folin, J. Biol. Chem., May, 1914, p. 487). The varying coefficients of diffusion of each, and the influence of the membrane, no doubt cause certain differences, and in the case of special substances greater differences may be caused by combination, either chemical or physical, with other constituents of the blood. In general the diffusate appears to resemble closely a diluted solution of the non-proteid substances of the blood, the dilution in the above one hour diffusate being about five to six times.

As the volume of the diffusate in this apparatus was about 4 to 5 times that of the blood in the tubes, it appears that the composition of the blood, which at the slowest probably refilled the tubes ten to twenty times[5] an hour, could have been changed only by a small percentage during its passage from the animal's artery back to the vein. Increase of surface in the apparatus, therefore, may be expected still to produce a nearly proportional increase in the quantity diffusing. A great increase may also be expected from efficient stirring of the outer fluid, as has been well established by Hess and McGuigan.[6]

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[4] Preliminary results indicate that this is also approximately true for creatin and creatinine, further results as to which are in progress.

[5] Viz: the total amount of blood which flowed through the apparatus in one hour was from twice to four times that of the diffusate, so that the amount removed by diffusion was only from one-tenth to one-twenty-fourth of the whole.

## II. OUTLINE OF METHOD OF SEPARATION OF THE CONSTITUENTS OF THE DIALYSATES

The hourly dialysates were brought to the boiling point for a moment immediately after being drawn off from the apparatus and were then concentrated on the water bath. To keep the temperature at a low point electric fans were made to blow across the evaporating surface. At night the dialysates were kept covered with toluene in well stoppered bottles. It will thus be seen that bacterial infection was entirely prevented.

As taken from the dialysing apparatus, the diffusates are faintly alkaline to litmus. During the evaporation hydrochloric acid is added from time to time and the reaction is kept neutral or slightly acid to litmus. When the sodium chloride has crystallized out to a large extent it is removed by filtration under pressure. At this stage of the evaporation a flocculent precipitate makes its appearance. This is removed with the salt. To the filtrate methyl alcohol is added and the salts thus thrown out are repeatedly exhausted with this solvent. The precipitated salts are then further exhausted with acidulated ($H_2SO_4$) ethyl alcohol of 95 per cent. The alcoholic extracts are combined, the alcohols removed by evaporation in vacuo, the residue diluted with much water, and brought to the neutral point by the addition of sodium carbonate.

At this point one may proceed to precipitate the "hexonebases" with phosphotungstic acid or one may first decompose the large amount of urea present by means of urease. In the latter case, which represents our more recent procedure, the steps to be taken are as follows:

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[6] See their paper entitled "The Condition of the Sugar in the Blood," to appear in the next issue of this journal (vol. vi, no. 1, Sept. 1914). In our previous paper (p. 300) we referred to the benefits of agitation of the outer fluid, which we accomplished by means of a rubber bulb attached to the outflow tube; but while fully aware of the advantage qualitatively, we had not worked out quantitatively the very remarkable difference so produced.

Since the urease is inhibited by much salt as well as by alcohol, care must be taken to keep the concentration of the former below 6 per cent. To allow for the possible inhibiting action of other substances, the urease was taken in larger proportion and allowed to act a longer time than in urine analysis. In one case for three litres of solution containing about 13 grams of urea we took 100 cc. of urease solution[7] and allowed the whole to stand for three days.

That this amount of urease does not introduce an appreciable quantity of nitrogenous impurities is shown by the following analysis. From 150 cc. of urease solution, after acting in the above manner, on pure urea (13 gms.) and subsequent precipitation with phosphotungstic acid, followed by the removal of ammonia, there was found only 58 mgm. of total nitrogen.

The ammonia produced by the urease is removed in the usual way, the solution is made acid (5 per cent) with $H_2SO_4$ and precipitated with phosphotungstic acid. The filtrate from this precipitate is freed from sulphuric and phosphotungstic acids by baryta and excess of the latter removed quantitatively by dilute $H_2SO_4$. At this point it is found that the solution when neutral to litmus still contains large amounts of barium, indicating the presence of other acids than the ordinary products of protein hydrolysis most of which are practically neutral to litmus. The solution is now evaporated in vacuo until nearly dry when alcohol is added and distilled off to remove most of the water. After repeating this process, fresh alcohol is added, anhydrous HCl gas is passed in nearly to saturation and the salt removed by filtration on the water pump, washing thoroughly with alcohol acidulated with gaseous HCl. The salt thrown out at this stage is very dark containing melanin-like substances produced by the action of the acid upon the carbohydrates present and possibly on other substances. The salt was found to retain 160 mgm. of nitrogen per 100 grams.

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[7] E. K. Marshall, Jr., Journ. of Biol. Chem., xiv, p. 283, 1913; xv, p. 487 and p. 95, 1913, and xvii, p. 351, 1914.

The esterification was now repeated twice in the usual manner, and the acid neutralized with the calculated quantity of sodium ethylate. The salt formed was filtered off and the solution evaporated in vacuo and the esters fractionated with the aid of a Gaede mercury pump.

The fractions obtained were unexpectedly small. When a temperature of 160°C. was reached, decomposition with evolution of large quantities of gas took place, so that the pressure could not be kept down below 6 mm. (Hg.) and the distillation had to be discontinued at 165°. It is probable that some amino-acids or other nitrogenous substances were decomposing as a strong odor of amines was detected at the outlet of the pump. We attribute this behavior to two causes. The large amount of dextrose and other non-volatile substances hinders the distillation of the amines. In future work it will be advisable to separate such substances previous to the esterification. The other cause was an unfortunate mistake in using alcohol in the process of esterification which was supposed to be absolute but which was found subsequently to have a strength of only 95 per cent. Considerable amounts of water were therefore present during the process of fractional distillation. The fractions obtained by vacuum distillation of the esters were saponified in the usual way. Attempts to isolate proline and phenyl-alanine led to negative results. It is probable that the less volatile amino-acids usually obtained in the higher fractions, were not distilled over, owing to the comparatively high pressure and low temperature attained, and also to the reduction of their vapour tension by the large quantity of sugar and other non-volatile substances present. The substances isolated are described in Section III. The further treatment of the residue is given on page 618.

## THE PHOSPHOTUNGSTIC PRECIPITATE

During April and May, 1913, the Kossel and Kutcher method of separating diamino-acids was applied to the small quantity of material then at our disposal. Qualitative tests which have been confirmed by this year's work indicated the presence of histidin.

All the fractions were apparently of composite character so that no definite substances were isolated. We are evidently dealing in this precipitate with an indeterminate number of substances. With the larger quantity of material obtained this year a different procedure was followed. The moist phosphotungstic precipitate was treated according to Wechsler's method,[8] being shaken for one and a half hours with a mixture of four parts of acetone and three parts of water. The soluble and insoluble portions were separately freed from phosphotungstic acid by baryta. The acetone insoluble portion contained relatively little organic nitrogen.

The acetone soluble portion after being freed from phosphotungstic acid was concentrated nearly to dryness and then taken up in methyl alcohol. After removing the alcohol, water was added and the solution precipitated with a concentrated aqueous solution of gold chloride. The amorphous precipitate obtained was decomposed with hydrogen sulphide and a small amount of an organic substance was obtained which has not yet been identified but which does not appear to be an amino-acid.

The filtrate from the gold chloride precipitate after being freed from gold and excess of hydrogen sulphide was neutralized with sodium carbonate and evaporated nearly to dryness in vacuo. The residue thus obtained was exhausted with methyl alcohol. After removal of the alcohol a syrup remains in which a deposit of crystalline character is slowly formed. The syrup gives Knoop's histidin test very distinctly. As already stated, the residue now under consideration contains the greater part of the nitrogenous substances precipitable by phosphotungstic acid.

## RESIDUE FROM THE DISTILLATION OF THE ESTERS

For reasons already stated a large part of the nitrogenous substances in the material subjected to fractional distillation in vacuo remained undistilled when the process was stopped at 165°. The residue was therefore separated into several fractions by repeated solution in alcohol and precipitation with ether, and those fractions which showed the larger amounts of nitrogen, whether total or amino-nitrogen, were separately treated. Thus there was obtained by precipitation by phosphotungstic acid from an intermediate fraction thrown out on adding two volumes of ether to an alcoholic solution of the residue, a fraction giving positive indications of creatinine by Jaffé's and Weyl's color reactions as well as by precipitation with aqueous picric acid.[9] There was also obtained from the fraction most soluble in ether, after removal of impurities by phosphotungstic acid, the crystalline ether-soluble substance melting at 211° referred to in section III, leaving a considerable amount of nitrogenous substances for further investigation. The crystalline body is precipitated by silver sulphate and baryta as well as by mercuric sulphate but not by picric acid. It was purified by crystallizing twice from a fairly concentrated solution in hot water. On cooling the substance is deposited in small glistening prisms arranged in feathery aggregates. It is freely soluble in alcohol, less soluble in cold water and fairly soluble in ether.

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[8] Zeitschr. f. physiol. Chemie, 73, 138, 1911.

The following analytical data were obtained with material twice recrystallized and melting at $210\frac{1}{2}$-211°C. (cor. .

1. 4.489 mgm.: 8.90 mgm. $CO_2$; 3.17 mgm. $H_2O$ = 54.13% C and 7.90% H
2. 4.252 mgm.: 8.41 mgm. $CO_2$; 2.96 mgm. $H_2O$ = 53.94% C and 7.79% H
3. 4.320 mgm. (724 mm., 19°C); 0.700 c.cm. N = 18.05% N

From these data the empirical formula, $C_7H_{12}N_2O_2$ is calculable.

| *Required for* $C_7H_{12}N_2O_2$ | *Found as above.* |
|---|---|
| C = 53.81 | C = 54.13; 53.94 |
| H = 7.75 | H = 7.90; 7.75 |
| N = 17.95 | N = 18.05 |

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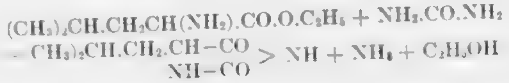
[9] This really indicates the presence originally of creatine, which was changed into creatinine, after the first phosphotungstic precipitation

The substance agrees in its properties with isobutyl-hydantoin (1. Isobutyl 2.4 diketo-tetrahydroimidazol) first prepared by Pinner and Lifschütz[10] and later by Fritz Lippich[11] from valeraldehydecyanhydrin and urea, also by E. Koenigs and B. Mylo from *dl.* Leucinamid and ethyl chlorcarbonate. The isolation of this substance does not necessarily prove its original presence in the blood of the dog, inasmuch as it may have been formed during the ester distillation by a condensation of urea with another substance. Leucine, which has not so far been isolated, but the presence of which seems probable as one of the commonest products of proteolysis, would have existed in the mixture, if at all, as leucine-ester. The similarity of the following hypothetical reaction, to that of Koenigs and Mylo on the one hand, and to that of Pinner, Lifschütz and of Lippich on the other will be obvious on inspection.



$$(CH_3)_2CH.CH_2CH(NH_2).CO.O.C_2H_5 + NH_2.CO.NH_2$$
$$= (CH_3)_2CH.CH_2.\begin{array}{l}CH-CO\\ \quad NH-CO\end{array} > NH + NH_3 + C_2H_5OH$$

Without laying too great stress on the above possibility we would leave it an open question, which we hope to investigate further, whether this and perhaps other hydantoin derivatives occur in the animal body.

## III

The following additional substances have been isolated up to the present time. Among amino acids only alanine and valine have been obtained in crystalline form. Crystals of alanine separated out in the summer of 1913 from fraction I of the vacuum distillation reported in an earlier paper. Alcohol and hydrochloric acid had been added to the fraction to cause the separation of glycine in the form of the hydrochloride of the ester. The latter substance was not obtained, but on long standing alanine was set free by the hydrolysis of its ester and crystallized out in the form of bunches of needles. In this year's work alanine was obtained in larger quantity, being the chief amino-acid in all four fractions. Besides portions left in the mother liquors, the amount of recrystallized pure alanine obtained was 0.330 gram.

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[10] Ber. d. d. Chem. Ges. 20, p. 2356 (1887).

[11] Ber. d. d. Chem. Ges. 41, p. 2972 (1908).

[12] Ber. d. d. Chem. Ges. 41, p. 4439 (1908).

It is noteworthy that no trace of glycine has been found either in the dialysates collected in 1912–13, or in those of the past year. The application of Levene's[13] picric acid method for separating alanin and glycin yielded only alanine, and at no time could we obtain crystals of glycin ester hydrochloride from the fraction which ordinarily contains the larger part of this ester.

Amino-acids of higher carbon content than alanine were sought for in the alcohol insoluble portions of the first three fractions obtained in the ester distillation. It may here be stated that the major part of these fractions is soluble in absolute alcohol and contains as will presently appear, considerable quantities of lactic, β-oxybutric and other acids. The alanine obtained from all of the fractions was recrystallized from very dilute alcohol and the amino-acid or acids remaining in the mother liquors were converted into the copper compound. This was dried in vacuo and then subjected to the solvent action of methyl alcohol, with the intention of effecting a preliminary separation, in case any appreciable amount of valin or other higher amino-acid was present. A little less than 0.1 gram of alcohol soluble copper compound was obtained. The copper content of the residue was found to agree with that required for valine.

Lactic acid was found in considerable quantity and analyzed in the form of its zinc salt. β–oxybutyric acid is also present in considerable quantity. Its presence was proved by the acetone formed on distilling with chromic acid mixture and redistillation with sodium hydrate and hydrogen peroxide.[14] Other acids, not amino-acids, are present in the product of the ester distillation. The presence of these oxy-acids is of particular interest from the point of view of carbohydrate metabolism. We recognize in this connection, that we were dealing with animals

[13] Journ. of Biol. Chem., xvi, p. 103, 1913.

[14] See Ph. A. Shaffer, Journ. of Biol. Chem., v, p. 211, 1908.

whose condition was abnormal in that they were deeply anaesthetized and towards the end of the experiment often showed a period of air hunger. How important these factors are in causing the appearance of these and other acids we can not pretend to say.

Creatinine has not been isolated in crystalline form but unmistakable evidence of its presence was obtained by the color reactions both of the original diffusate and of the residue of the distillation of the amino esters. Its presence with the substances not precipitated by phosphotungstic acid indicates the original presence of creatin which was changed into creatinine during esterification.

The presence of histidin among the diamino-acids was determined by Knoop's color reaction as already stated. Many other substances undoubtedly remain to be isolated from the various fractions which are still in the course of separation.

## ANALYTIC DATA FOR SUBSTANCES OBTAINED IN THE ESTER-DISTILLATION

The microchemical analyses in this paper were made by Dr. Hans Lieb in Professor Pregl's laboratory in Graz. We take pleasure in recording here our indebtedness to him for the skillful manner in which they were carried out, and cannot too strongly recommend to biochemical investigators the usefulness and accuracy of the methods devised by Professor Pregl. Without them, the small quantity of one of the substances which we have isolated could not have been identified.

The alanine analysed was obtained as already stated from Fraction I in the ester distillation of May 1913, and purified by recrystallization.

1. 4.040 mgm. with an ash content of 0.004 = 4.036 mgm. of ash free substance, gave 2.845 mgm. $H_2O$ and 5.99 mgm. $CO_2$ = 7.89 per cent H and 40.48 per cent C.

2. 4.286 mgm. with an ash content of 0.003 mgm. = 4.283 mgm. of ash free substance, gave 3.11 mgm. $H_2O$ and 6.335 $CO_2$ = 8.13 per cent H and 40.34 per cent C.

3. 2.966 mgm. of substance gave 0.404 cc. N, barometric pressure, 741 mm. and t = 21°C, = 15.42 per cent N.

4. 4.476 mgm. of substance gave 0.606 cc. N, barometric pressure 742 mm. and t = 21°C. = 15.35 per cent N.

| *Found* | | *Required for Alanine,* $C_3H_7NO_2$ |
|---|---|---|
| C = 40.48; | 40.34 | C = 40.42 |
| H = 7.89; | 8.13 | H = 7.92 |
| N = 15.42; | 15.35 | N = 15.73 |

These data taken in connection with the manner in which the substance was obtained (ester distillation), and with the fact that it gives the ninhydrin reaction, is practically insoluble in alcohol, and gives up its nitrogen on being decomposed with nitrous acid make it evident that the substance here described is $\alpha$–amino-propionic acid, or alanine, $C_3H_7NO_2$.

It was not thought necessary to subject the corresponding substance obtained in this year's work to an elementary analysis, but the following corrected decomposition points on rapid heating were found for five specimens of crystallized alanin obtained from the different fractions of the ester distillation, 295°C, 292°C, 282°C, 283°C, 286°C.

Valine. The copper salt as obtained from its solution in methyl alcohol (99 per cent) weighed 0.0906 gm. (dried at 110°C) and yielded 0.0285 gm. CuS = 0.0190 gm. Cu = 20.97 per cent while the anhydrous copper salt of valin, $(C_5H_{10}NO_2)_2$ Cu, requires Cu = 21.51 per cent. After removing the copper, the free valine was crystallized from water.

Lactic acid. As already stated this acid appears in considerable quantity among the products of the ester-distillation. From fraction I, for example, we separated 1.75 grams of ether soluble acids consisting largely of lactic and $\beta$-oxybutyric acids. The zinc salt of lactic acid was purified by recrystallization from water.

0.1439 gram salt on ignition after treatment with nitric acid gave 0.0412 gram ZnO = 23.01 per cent Zn.

0.1439 gram salt lost at 105–110°C., 0.0212 gram water = 14.71 per cent.

| $Zn(C_3H_5O_3)_2.2H_2O$ *requires* | *Found as above.* |
|---|---|
| 12.86 per cent $H_2O$ | 14.71 per cent $H_2O$ |
| 23.41 per cent Zn | 23.01 per cent Zn |

The analytical data here given show that we are dealing with the zinc salt of paralactic acid and confirm earlier analyses as of Gaglio[15] that lactic acid is a normal constituent of the blood of the dog. The high value for water in the above analysis is probably due to a partial racemization. The salt of the inactive acid crystallizes with three molecules of water and contains 18.12 per cent and 21.99 per cent Zn. Calculated as percentage of the anhydrous salt the above analysis gives 26.98 per cent Zn found, as against 26.87 per cent required by theory.

## SUMMARY

1. The non-protein constituents of the blood have been accumulated in considerable quantity by our method of vividiffusion. The total amount of non-protein nitrogen obtained in one hundred and twelve hours was about 20 grams. Other substances, especially sugar dialysed in large quantities.

2. Quantitative data are given on the rate of accumulation of different nitrogenous substances and it is shown that their relative proportions in the diffusate do not vary very greatly from those in the blood.

3. Alanine and valine have been obtained in crystalline form. Histidine and kreatinine have been shown to be present by reactions.

4. Oxyacids were found in noticeable proportion. Lactic and β-oxybutyric acids in particular have been identified.

5. In the residue from the ester-distillation there was found a crystalline substance having the composition, $C_7H_{12}N_2O_2$. In its melting point (210½ to 211° cor.), and solubilities it agrees with α-isobutyl hydantoin. The question whether this substance exists as such in the blood or whether it has been formed by chemical action in the process of isolation has not yet been decided.

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[15] Arch. f. (Anat. u.) Physiol., 1886.

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